

12

**EUROPEAN PATENT APPLICATION**

21 Application number: **84201301.3**

51 Int. Cl.<sup>4</sup>: **C 12 N 9/30**  
**C 12 N 9/34, C 12 P 19/20**

22 Date of filing: **10.09.84**

30 Priority: **11.09.83 EP 83201303**

43 Date of publication of application:  
**08.05.85 Bulletin 85/19**

84 Designated Contracting States:  
**AT BE CH DE FR GB IT LI LU NL SE**

71 Applicant: **Gist - Brocades N.V.**  
**Wateringseweg 1 P.O. Box 1**  
**NL-2600 MA Delft(NL)**

72 Inventor: **Ducroo, Paul**  
**Rue Mermoz 6**  
**F-59133 Phalempin(FR)**

72 Inventor: **Labout, Johannes Jacobus Maria**  
**Alberdingk Thijmalaan 8**  
**NL-2106 EK Heemstede(NL)**

72 Inventor: **Noordam, Bertus**  
**Wagenaarhof 19**  
**NL-2692 BJ 's-Gravenzande(NL)**

74 Representative: **Huygens, Arthur Victor, Dr. et al,**  
**c/o Gist-Brocades N.V. Patent & Trademarks Department**  
**Wateringseweg 1 PO-box 1**  
**NL-2600 MA Delft(NL)**

54 Novel enzyme product and its use in the saccharification of starch.

57 A novel acid amylase is provided, isolated from amyloglucosidase, having a greater thermostability than amyloglucosidase and a comparable pH-optimum. This acid amylase can be used advantageously in conjunction with amyloglucosidase and optionally with an acid pullulanase in the saccharification of starch and starch hydrolysates. Also, a novel enzyme product is provided comprising acid amylase having  $\alpha$ -1, 4-glucosidic bond splitting activity and amyloglucosidase and optionally acid pullulanase.

NOVEL ENZYME PRODUCT AND ITS USE IN THE SACCHARIFICATION OF STARCH

FIELD OF THE INVENTION

5

The present invention relates to enzymatic starch degradation. More specifically, the invention provides a novel enzyme product useful in the saccharification of starch, especially liquefied starch, and a process for its  
10 preparation.

STATE OF THE ART

Native starch is known to contain two types of  
15 macromolecules composed of glucose units. One type of molecule, called amylose, is linear and consists exclusively of  $\alpha$ -1,4-linked glucose units. Starch contains about 25% of amylose. The second type of molecule, called amylopectin, is highly branched and contains  $\alpha$ -1,4 as well as  $\alpha$ -1,6 linked  
20 glucose units. The overall content of  $\alpha$ -1,6 linkages is generally less than 5%.

Sugars from starch, in the form of concentrated dextrose syrups, are currently produced at the rate of several million tons per annum by a two stage enzyme catalyzed process  
25 involving: (1) liquefaction (or thinning) of solid starch with an  $\alpha$ -amylase into dextrans having an average degree of polymerization of about 7-10, and (2) saccharification of the resulting liquefied starch (i.e. starch hydrolysate) with amyloglucosidase, which results in a syrup of high glucose  
30 content (92-96% by weight of the total solids). Much of the dextrose syrup produced commercially is then enzymatically isomerized to a dextrose/fructose mixture known as isosyrup.

The two enzymes used,  $\alpha$ -amylase and amyloglucosidase, differ in two important aspects. First,  $\alpha$ -  
35 amylase, which is a so-called endo-enzyme, attacks macromolecules at random. Amyloglucosidase, on the other hand, is a so-called exo-enzyme and splits glucose units successively from the non-reducing end of the dextrin molecule in the

starch hydrolysate. Secondly,  $\alpha$ -amylase exclusively attacks  $\alpha$ -1,4 linkages whereas amyloglucosidase splits  $\alpha$ -1,6 linkages as well.

The recommended name of amyloglucosidase is exo-1,4-  
5  $\alpha$ -D-glucosidase, the Enzyme Committee number 3.2.1.3 and the systemic name  $\alpha$ -1,4-glucan glucohydrolase. Amyloglucosidase is also called AG or glucoamylase and it will be understood that the terms amyloglucosidase, AG and glucoamylase, as used hereinafter, are synonymous.

10           Whereas amylopectin is only partially degraded by  $\alpha$ -amylase because this enzyme exclusively attacks  $\alpha$ -1,4 linkages, substantial hydrolysis of the branched oligosaccharides occurs in the subsequent saccharification step catalyzed by amyloglucosidase which also hydrolyses  $\alpha$ -1,6  
15 glucosidic linkages, though at a considerably lower rate than the  $\alpha$ -1,4 linkages.

The saccharification stage of the commercial process outlined above has long been recognized to be deficient in certain regards. In particular, the amyloglucosidases  
20 currently available catalyse both saccharification and dextrose reversion reactions, e.g. conversion of dextrose into isomaltose, at rates which depend on the substrate concentration. The formation of by-products in this way has limited the saccharification of starch hydrolysates into  
25 dextrose to not more than about 95% by weight of dextrose on dry solids basis (hereinafter termed DX) in syrups containing at least 33% dry solids by weight.

It is true that the formation of by-products from reversion reactions may be suppressed by up to about 50% with  
30 a concomitant increase of starch conversion of about 1-2% if a relatively high level of amyloglucosidase combined with a dilution of the substrate to about 15% dry solids is employed (cf. U.S. Patent No. 4,017,363), but the concentration of the resulting dextrose solution to the conventional higher dry  
35 solids levels is energy consuming.

In an effort to further increase the DX value it has been proposed to use a debranching enzyme, in conjunction with amyloglucosidase, so as to hydrolyze more efficiently the branched oligosaccharides (containing  $\alpha$ -1,6 glucosidic bonds)

present in the liquefied starch.

European Patent Application No. 82302001.1,  
Publication No. 0 063 909, describes a debranching enzyme of  
the pullulanase type which is produced by a Bacillus called  
5 Bacillus acidopullulyticus. According to this specification  
the debranching enzyme has optimum activity at a pH in the  
range of 3.5 to 5.5 (under defined conditions) and its thermal  
activity optimum at pH 4-5 is at least about 60°C. The  
residual activity after 72 hours at 60°C at pH 5 is 50% or  
10 more. This acid pullulanase is used together with one of the  
saccharifying enzymes amyloglucosidase or  $\beta$ -amylase. The use  
of this acid pullulanase in conjunction with amyloglucosidase  
is reported to result into a higher dextrose level which is  
higher by about 1% as compared with the level obtained with  
15 amyloglucosidase alone under similar conditions. Alternatively  
the same dextrose level may be achieved using about half the  
amount of amyloglucosidase.

U.S. Patent No. 4,335,208 discloses the combined  
action of amyloglucosidase and another debranching enzyme,  
20 namely isoamylase from Pseudomonas amyloclavata. According to  
this reference the isoamylase has a pH optimum close to that  
of amyloglucosidase so that the amount of the latter can be  
considerably reduced to obtain the same or even a higher  
dextrose level than with amyloglucosidase alone. However, the  
25 process has a serious drawback in that the isoamylase is heat  
labile. This means that no saccharification in the presence of  
isoamylase is technically feasible above about 55°C, whereas  
amyloglucosidase by itself is normally used at 60°C in the  
saccharification of starch hydrolysate. Moreover, micro-  
30 organisms of the genus Pseudomonas are not so-called GRAS-  
microorganisms (Generally Regarded As Safe), so that enzymes  
produced by such microorganisms are not permitted in food and  
food processing in the USA.

U.S. Patent No. 3,897,305 discloses the combined use  
35 of amyloglucosidase and pullulanase from Aerobacter aerogenes  
(Klebsiella pneumoniae) which is stated to give an increase in  
DX of up to 2% in syrups containing at least 30% dry solids.  
Practically no saving of amyloglucosidase is achieved however,  
because of the unfavourable pH optimum (5.5-6.0), of the

enzyme from K. pneumoniae which makes it necessary to conduct the saccharification at a relatively high pH at which the activity of amyloglucosidase is severely reduced.

Marshall et al (Febs Letters, Vol. 9 No. 2, July 5 1970, pages 85-88) reported that amyloglucosidase obtained from Aspergillus niger contained an  $\alpha$ -amylase-like impurity apparently essential for complete hydrolysis of starch to glucose. No attempt was however made to characterize or isolate this impurity.

10

#### OBJECTS OF THE INVENTION

It is an object of the invention to provide a novel acid amylase, which can be derived from amyloglucosidase 15 preparations, having substantial  $\alpha$ -1,4-glucosidic bond splitting activity. The novel enzyme product has  $\alpha$ -glucosidic bond splitting activity at acidic pH, and can be used in the saccharification of starch and, preferably, liquefied starch.

It is a further object of the invention to provide a 20 novel process for converting starch into syrups with a high dextrose content.

#### THE INVENTION

25 According to its first aspect the present invention provides a microbial acid amylase, obtainable from amyloglucosidase, and having substantial  $\alpha$ -1,4-glucosidic bond splitting activity. This acid amylase effects optimum saccharification at a pH between 3.5 and 5.0 at temperatures 30 from about 60 to about 75°C. Under ordinary storage conditions it is stable over a period of several months.

The acid amylase of the invention occurs as a component in amyloglucosidase preparations and can be obtained in substantially pure form from such preparations using an 35 appropriate separation technique, such as high performance liquid chromatography which is also the preferred method.

Although the novel acid amylase described below is obtained from a commercially available amyloglucosidase derived from the microorganism Aspergillus niger, and this is

the preferred amyloglucosidase, it will be appreciated that many genera of microorganisms contain species known to produce an amyloglucosidase. Any and all such amyloglucosidases can be used as the source of the novel acid amylase of this invention. Preferably, a fungal amyloglucosidase is used as the source.

The thermostability of the acid amylase derived from Aspergillus niger is better than that of the A. niger amyloglucosidase. Also the stability and residual activity of said acid amylase exceeds the same of said amyloglucosidase.

The invention further provides a novel enzyme product having both  $\alpha$ -1,4 and  $\alpha$ -1,6 bond splitting activity at acidic pH, which comprises amyloglucosidase and the novel acid amylase in a ratio of at least 0.16 AAU per AGI, as herein-after defined.

Such preparations may be made by adding the novel acid amylase to a known amyloglucosidase preparation so as to increase the acid amylase content of the latter.

Preferably, the amyloglucosidase is an Aspergillus niger amyloglucosidase and is enriched with the novel acid amylase also derived from Aspergillus niger.

The novel enzyme product of the invention can be prepared by adding the new acid amylase, preferably in substantially pure form, to the amyloglucosidase. Alternatively, an amyloglucosidase producing strain or variant or mutant thereof, preferably belonging to the genus Aspergillus and more preferably to the species A. niger, may be found which produces an amyloglucosidase with a relatively high acid amylase content as compared with the amyloglucosidases known in the art, in which case the enzyme product may be obtained by cultivating the said microorganism in a suitable nutrient medium containing carbon and nitrogen sources and inorganic salts. The novel enzyme product may also be prepared by selectively improving the fermentation conditions for acid amylase or partly inactivating the amyloglucosidase in existing preparations.

The amyloglucosidase used in this invention and also the novel enzyme product are preferably free from trans-glucosidase, since the latter enzyme may cause the formation

of undesired by-products. This can be effected e.g. by producing amyloglucosidase with a transglucosidase-negative strain or by removal of the transglucosidase from the amyloglucosidase preparations used, for example with bentonite.

5           The novel enzyme product of the invention comprises at least 0.16 AAU of acid amylase per AGI. One unit of acid amylase activity (AAU) as used herein is the amount of enzyme which hydrolyses 1.0 mg of soluble starch (100% of dry matter) per minute under standard conditions (pH 4.2; 60°C) into a  
10 product which, after reaction of an iodine solution of known strength, gives an optical density at 620 nm equivalent to that of a colour reference as described in the Iodine Starch Amylase Test described below. One unit of amyloglucosidase activity (AGI) as used herein is defined as the amount of  
15 enzyme that releases 1  $\mu$ mol of dextrose from soluble starch (100% of dry matter) per minute at 60°C under optimum conditions of starch degradation, as described hereinafter. Preferably, the novel enzyme product contains from about 0.2 to about 4.5 AAU of acid amylase per AGI, more preferably from  
20 about 0.3 to about 3.0 AAU per AGI and particularly from about 0.7 to about 1.5 AAU per AGI.

It has been surprisingly found that the amyloglucosidase preparations enriched with acid amylase, when used in the saccharification of liquefied starch, result in  
25 unexpectedly and significantly higher dextrose levels at shorter saccharification times. The results are comparable with those obtained by the simultaneous action of amyloglucosidase and acid pullulanase, as described in the aforementioned European Patent Application Publ. No. 0 063 909,  
30 under similar conditions.

Accordingly, the invention further provides a process for converting starch into dextrose in the form of a syrup, which comprises saccharifying the starch optionally and preferably after a liquefaction step to form a starch  
35 hydrolysate, in the presence of the novel enzyme product, as hereinbefore defined. The use of the new enzyme product in the process has the advantage that substantially lower amounts of amyloglucosidase can be used for saccharification of starch hydrolysates resulting in higher yields of glucose per enzyme

unit (AGI). The new enzyme product has also the great advantage that higher substrate concentrations can be used in the saccharification of starch and starch hydrolysates. The use of higher substrate concentrations substantially reduces 5 evaporation costs.

The saccharification is suitably carried out at a pH in the range of from 2.5 to 6, preferably of from about 3 to about 5 and more preferably of from about 4.0 to about 4.5. The process is suitably effected at temperatures in the range 10 of from 40 to 70°C, preferably of from about 50 to about 65°C, with reaction times in the range of from 15 to 96 hours to obtain maximum yields.

Preferred proportions of amyloglucosidase for the saccharification of starch hydrolysates are normally in the 15 range of from about 8 to about 30 AGI and preferably from about 14 to about 22 AGI per g of dry solids.

It has also been found that the saccharification of starch or a starch hydrolysate can be further improved, when the process is conducted in the presence of the novel enzyme 20 product as defined hereinbefore, which also contains an effective amount of acid pullulanase. A suitable acid pullulanase which can be used for the purpose of this invention is, for example, an acid pullulanase as described in European Patent Application Publ. No. 0 063 909. Preferred 25 dosages of acid pullulanase which can be used in conjunction with the novel enzyme product are in the range of from 0.005 to 5 pullulanase units (PU), the units being as defined in said European Patent Application. The use of the novel enzyme in conjunction with acid pullulanase in the process has the 30 advantage that unexpectedly and significantly high dextrose levels can be obtained at short saccharification times.

Another suitable method to determine the amount of acid amylase in enzyme preparations is the modified Phadebas Amylase Test described below. One unit of acid amylase 35 activity (AAU') as used herein is defined as the amount of enzyme that gives one unit of absorbance at 620 nm under modified Phadebas amylase test conditions described below. The value of at least 0.16 AAU of acid amylase per AGI under Iodine Starch Amylase Test conditions, as defined herein-



before, corresponds with the value of at least 0.12 AAU' of acid amylase per AGI under modified Phadebas Amylase Test conditions. A drawback of the latter method is a synergistic effect which occurs when amyloglucosidase is present.

5 Moreover, it is very difficult or even impossible to automatize this method.

It will be understood that, unless otherwise stated, the AAU values which are mentioned in this specification are expressed in units according to the modified Iodine Starch  
10 Amylase Test method.

The following test methods and Examples illustrate the invention.

#### IODINE STARCH AMYLASE TEST

15

This method is based on the measurement of the optical density of iodine starch complexes in the presence of an amyloglucosidase inhibitor. Acarbose, Bay g 5421, was used as the amyloglucosidase inhibitor, cf. Schmidt et al.,

20 Naturwissenschaften 64 (1977) 535.

#### Reagents

- A 2% solution of soluble starch (Lintner, J.T. Baker Co.) in  
25 citrate buffer (0.013 M, pH 4.2).

- Iodine stock solution containing 22 g iodine and 44 g potassium iodide per litre of distilled water.

30 - Diluted iodine solution: 4 ml of iodine stock solution and 40 g potassium iodide dissolved in distilled water. Distilled water added up to 1 litre.

- Colour reference containing 250 g cobaltous chloride 6 aq.  
35 and 38.4 g potassium bichromate per litre in 0.01 N HCl.

#### Procedure

The starch solution (20 ml) was preheated at 60°C

for 20 min. Starting at time 0 exactly 10 ml of the enzyme sample (containing 1.4 - 1.8 AAU/ml; room temperature) was added to the substrate solution. If amyloglucosidase is believed to be present in the enzyme sample, the amylo-  
5 glucosidase inhibitor Bay g 5421 is previously added to the enzyme sample in a concentration of 1 ug per AGI. After 20 min. of incubation 1 ml of the solution was transferred to 5 ml of the diluted iodine solution. The optical density was immediately measured at 620 nm in a 1 cm cuvet using distilled  
10 water as the blank. This procedure of transferring and measuring was repeated at 1 min. intervals until readings were found which were lower than the readings of the colour reference.

15 The time T needed to reach the absorbance equal to that of the colour reference was established graphically.

The acid amylase activity in units (AAU) present in the incubation solution was calculated from  $400/T$  in which:  
20 400: mg of soluble starch in the incubation solution  
T : reaction time needed (min.)

#### MODIFIED PHADEBAS AMYLASE TEST

25 The standard Phadebas amylase test (Marciniak et al., Starch 34 442 (1982)) modified for conditions of acidic pH and a temperature of 60°C was effected as follows. In a glass vial with screwed cap 1 ml of enzyme sample containing 10 AGI and 4.0 ml acetate buffer (0.3M, pH 4.0) were pipetted.  
30 Then a Phadebas tablet (Pharmacia, batch no. HE 74112) was added and after vortexing for 15 sec. the tube was closed and placed in a water bath at 60°C. The reaction was stopped exactly 15 min. after the addition of the tablet by adding 0.3 N NaOH (5 ml) and shaking. After centrifugation the super-  
35 natant was removed and the optical density (OD) was measured (in the range 0.2 to 2.0) in a 1 cm cuvet at 620 nm relative to distilled water. A blank (distilled water) underwent the same procedure. The  $\Delta OD$  is a measure of the acid amylase activity. One unit of acid amylase activity (AAU') is defined

as the amount of enzyme that gives one unit of absorbance ( $\Delta OD = 1$ ) at 620 nm under these test conditions.

#### AMYLOGLucosIDASE ASSAY

5

Soluble starch (2 ml; Lintner Starch, J.T. Baker Co.) in a concentration of 16 g/l of acetate buffer (0.04M, pH 4.3) was preheated at 60°C for 5 min. and then added to 2 ml of enzyme solution (0.15-0.55 AGI/ml). After mixing the  
10 suspension was incubated at 60°C. The reaction was terminated after 15 min. by adding 20 ml NaOH (0.005 N) and the glucose concentration determined by the glucose oxidase method.

#### THE SACCHARIFICATION PROCESS

15

The saccharification process was effected on malto-dextrin MDO3 (Roquette Frères) having a dextrose equivalent (DE) of 16.5. This substrate contains some oligosaccharides having fructosyl end groups, from which as much as 0.4-0.5% of  
20 the disaccharide maltulose is formed in the saccharification step. To a solution of this substrate (33% dry solids) 2100 AGI/100 g dry solids were added. The pH was adjusted to 4.20 with 1 N acetic acid. The mixture was incubated at 60°C in a water bath. Aliquots of 0.1 ml were taken from the reaction  
25 mixture at 16, 24, 48, 64, 72, 80 and 92 hr and added to 3 ml of distilled water in a closed test tube.

Each diluted sample was immediately placed into a boiling water bath for 10 min. in order to inactivate the enzyme. After cooling about 150 mg of dried Amberlite MB-3  
30 resin (BDH) were added to each sample in order to remove HPLC disturbing salts. After standing for 1 hr the resin was removed and 40  $\mu$ l of sample were injected onto the HPLC for glucose determination according to the method of Scobell et al. (Cereal Chem., 54 (4), (1977) 905-917), modified in that a  
35 Bio-Rad HPX-87C 300 mm column was used. The precision and accuracy of the assay were found to be 0.1% and 0.2% absolute respectively at a glucose concentration in the range of 90-96%.

Under these conditions a peak level of 94.6-94.8% of

glucose was achieved using current commercial amyloglucosidase preparations from Miles (DIAZYME and OPTIDEX), Novo (AMG) and Gist-Brocades (AMIGASE GM).

5

EXAMPLE IIsolation and Identification of Acid Amylase

In order to identify and isolate amylolytic components present in an amyloglucosidase preparation, an amyloglucosidase enzyme preparation produced by a trans-glucosidase negative strain of A. niger was subjected to high performance liquid chromatography. The system comprised an anion exchange column and a gel filtration column coupled in series. After injection of a part of the AG preparation onto the ion exchange column, the solvent, which was 0.05 M sodium acetate buffer with a pH of 4.0, was led through both columns until the positively charged and uncharged components had reached the gel column. The molecules adsorbed on the ion column were eluted by a salt gradient (0.05 - 1.65 M sodium acetate buffer, pH 4.0) and then the molecules bound on the gel column were eluted with the original solvent.

This procedure revealed an excellent separation as can be seen from the accompanying Fig. 1, between the amylolytic enzymes, and both amyloglucosidase isomers and an  $\alpha$ -amylase were identified by fractionating the effluent and incubating the collected fraction with suitable substrates, i.e. maltose and soluble starch (10% dry solids). The isolated amyloglucosidase components produced glucose from starch and maltose, whereas the isolated  $\alpha$ -amylase produced a typical oligosaccharide pattern from starch.

The characteristics of the  $\alpha$ -amylase, in particular in relation to pH and temperature, were determined using soluble starch as a substrate. The amounts of the main products formed (di- and trisaccharides) showed an optimum at a pH of 3.5 to 5.0 indicating that the enzyme is a true acid amylase (AA). The effect of temperature was investigated at a pH of 4.0 and the acid amylase had its optimum between 65 and 70°C as was determined from the behaviour of the tri-

saccharides formed. These results indicate that the acid amylase is sufficiently stable at the standard saccharification temperature of 60°C. The fractions containing AA, the activity of which was stable for more than 3 months, 5 were used for enrichment experiments.

#### EXAMPLE II

##### Saccharification with Acid Amylase Enriched Samples

10

Saccharification was accomplished on maltodextrin MDO3 having a dextrose equivalent (DE) of 16.5, and, to a solution of this substrate (33% dry solids), 2100 AGI/100 g dry solids were added. Also, various amounts of acid amylase 15 were added, the activity of which was previously determined according to Phadebas method described above. During the saccharification, the starch hydrolysate was maintained at a pH of 4.0-4.2 and a temperature of 60°C. Under these conditions the degree of saccharification or glucose formation 20 was measured over the period between 17 and 91 hrs.

The experiments with acid amylase enriched samples which were completely free of transglucosidase demonstrated that the glucose yield was increased and the saccharification time was shortened as can be seen from the results of Table I below.

0140410

TABLE I

					Glucose yield (%)							
Sample No.	AAU'/AGI (a)	AAU'/AGI (b)	AAU/AGI (c)	Saccharification time in hr.								
				17	24	41	48	65	72	91		
1	0.08	--	0.074	89.1	92.5	94.1	94.1	94.7	94.7	94.7	94.5	
2	0.08	0.07	0.20	90.7	93.6	94.6	94.8	94.7	94.7	94.7	94.5	
3	0.08	0.15	0.35	91.7	94.2	94.9	94.8	95.0	94.8	94.8	94.6	
4	0.08	0.35	0.73	92.7	94.6	94.9	94.9	94.6	94.8	94.8	94.5	
5	0.08	0.70	1.40	93.5	95.0	94.8	94.8	95.0	94.8	94.8	94.2	
6	0.08	1.50	2.92	94.0	94.9	94.8	--	94.4	94.4	94.4	94.3	
7	0.08	2.32	4.48	94.4	95.1	94.7	--	95.0	94.5	94.5	94.4	

(a): present in original amyloglucosidase preparation (measured with modified Phadebas method)

(b): AAU' of added acid amylase (measured with modified Phadebas method)

(c): acid amylase activities converted into AAU (as defined hereinbefore in the Iodine Starch Amylase Test)

The results of Table I show that acid amylase increases the yield of glucose from 94.7% to 95.1% under these conditions while the saccharification time for optimum yields decreased from 70 hours to 24 hours. This makes the use of acid amylase commercially important and equivalent to the results obtained with pullulanase. A portion of the amyloglucosidase can be replaced with the acid amylase while obtaining economically attractive glucose yields and saccharification times.

10

EXAMPLE III

Using the saccharification procedure of Example I, tests were run with the normal and one half normal dosages of amyloglucosidase and with one half normal dosages of amyloglucosidase with enrichment of acid amylase and acid pullulanase, described in European Patent Application Publication No. 0 063 909, alone and in combination. 1 pullulanase unit (PU) is defined as the amount of enzyme necessary to produce 1  $\mu$ mole of reducing sugar from pullulan per minute under standard conditions. The results are reported in Table II.

TABLE II

						Glucose yield (%)						
Sample No.	AGI (relative dosage)	AAU' / AGI (a)	AAU' / AGI (b)	AAU / AGI (c)	PU/gDS	Saccharification time in hr.						
						17	24	43.5	48	65	71	89
1	1.0	0.08	--	0.074	---	90.8	92.3	94.3	94.4	94.6	94.7	94.7
2	0.5	0.08	--	0.074	---	80.1	87.0	91.0	91.8	93.1	93.7	94.4
3	0.5	0.08	0.88	1.74	---	85.4	91.4	94.6	94.5	95.0	95.0	95.3
4	0.5	0.08	0.88	1.74	1.2	87.3	93.5	95.5	95.4	95.5	95.7	95.5
5	0.5	0.08	1.68	3.26	---	88.1	93.3	94.9	95.0	95.1	95.4	95.6
6	0.5	0.08	--	--	1.2	83.4	90.4	95.2	95.4	95.5	95.5	95.7
7	0.5	0.08	1.68	3.26	1.2	90.3	94.2	95.4	95.4	95.4	95.3	95.5

(a): present in original amyloglucosidase preparation (measured with modified Phadebas method)

(b): AAU' of added acid amylase (measured with modified Phadebas method)

(c): acid amylase activities converted into AAU (as defined hereinbefore in the Iodine Starch Amylase Test)



The results of Table II show that the glucose level reached its peak after about 80 hours using half the normal amyloglucosidase (AG) and an acid amylase (AA) enrichment factor, which is slightly longer than the 70 hour saccharification time using the normal amyloglucosidase dosage. However, the peak level of glucose increased from 94.7% to 95.5% which may be due to less isomaltose formation consequent upon the smaller amount of amyloglucosidase used.

Similar results were obtained when pullulanase and amyloglucosidase were used together, although significant differences in glucose production were noted at the shorter saccharification times. The combination of acid amylase, pullulanase and one half of amyloglucosidase showed a faster saccharification resulting in higher yields of glucose per enzyme activity (AGI) per hour.

These results indicate that acid amylase substantially contributes to the hydrolysis of starch in the saccharification step. This surprising effect competes with that of the acidic pullulanase, although the two enzymes act by basically different mechanisms. While pullulanase is thought to be an endo  $\alpha$ -1,6 bond splitter, acid amylase has  $\alpha$ -1,4 bond splitting activity.

#### EXAMPLE IV

Using the novel acid amylase in the saccharification of starch makes possible an increase in the glucose peak levels, a shortening of the saccharification times and a reduction of the necessary amyloglucosidase/dry solids (DS) ratio. Another advantage in using acid amylase in the saccharification of liquefied starch is the increase in substrate concentration which is then possible, which can substantially reduce evaporation costs.

Solutions containing substrate (MD03) in various dry matter contents were adjusted to pH 4.2 and heated to 60°C. Half normal AG dosages (10.5 AGI/gDS) and a 9-fold amount of acid amylase were added. Aliquots were taken at various intervals and analyzed as described in Example I. Control experiments with normal and halved AG dosages without

additional acid amylase were also carried out. The results are given in Table III below.

TABLE III

	%DS*)	AGI/gDS	AAU/AGI	Saccharification time (h)	maximum glucose(%)
5	25	10.5	0.074	140-165	95.6
	25	10.5	0.74	64	96.1
10	29	10.5	0.074	140-165	95.3
	29	10.5	0.74	90	95.8
	33	10.5	0.074	140-165	94.6
	33	10.5	0.74	90	95.2
	37	10.5	0.074	140-165	93.9
15	37	10.5	0.74	71	94.7
	45	10.5	0.074	140-165	91.3
	45	10.5	0.74	71	92.8
	33	21	0.074	71	94.7

20 \*) maltodextrin MD03

The data in Table III show that when amylo-glucosidase is used in conjunction with the new acid amylase the dry matter content (DS) can be elevated to yield maximum glucose levels which are higher than those obtained under similar conditions using commercial amyloglucosidase preparations. For example, a glucose peak level of 94.7% was obtained with a commercial amyloglucosidase preparation at 33% DS. The same maximum glucose level was achieved at the same incubation time with a 10 fold addition of acid amylase and half the amount of AG at 37% DS.

#### EXAMPLE V

35 Acid  $\alpha$ -amylases from other sources, i.e. bacterial enzymes, which are active in the acidic pH range and at 60°C, can also be used to improve the saccharification brought about by amyloglucosidase. Thus a crude fermentation sample of the bacterium ATCC 31199 (see British Specification No. 1539694

CPC International), containing  $\alpha$ -amylase activity, was used in a saccharification experiment with amyloglucosidase. Using the crude sample in a ratio of AAU/AGI = 0.74 gave significant higher glucose levels compared with those obtained with amylo-  
5 glucosidase only in a control experiment, although the values were lower than those obtained by a corresponding amount of the fungal acid amylase. The results are shown in the following Table IV.

TABLE IV

			Glucose yield (%)									
Sample	AAU/AGI	AGI/gDS	Saccharification time in hr.									
			24	40	47	64	71	88	95	112		
AG	0.074	10.5	80.2	88.9	90.3	92.2	92.8	93.6	94.0	94.3		
AG + Bacterial AA	0.74	10.5	81.7	90.4	91.6	93.1	93.5	94.1	94.2	94.6		
AG + Fungal AA (cf. Example 1)	0.74	10.5	--	94.4	94.7	95.1	95.2	95.2	--	--		

EXAMPLE VI

Saccharification experiments were performed by the same procedure as described hereinbefore. Solutions containing 5 substrate (MD03, 33% DS) were adjusted to pH values between 3.5 and 5.0 and heated to 60°C. Amyloglucosidase (21 AGI/g DS) together with a 9-fold amount of AA (compared with the amount present in the AG preparation) were added. Aliquots were taken at various intervals and analyzed. Control experiments were 10 also performed. The following data were obtained, see Table V.

TABLE V

	Starting pH	pH after 94 h	saccharification time (h)	glucose peak level (%)
15	3.5	3.45	71	95.3
	4.0	3.85	64	95.3 (94.9)*
	4.2	3.95	64	95.3 (94.8)*
	4.5	4.1	64	95.3 (94.6)*
20	5.0	4.2	64	95.2

\* Controls (AG dosages without extra AA addition)

Thus, using excess of acid amylase comparable 25 glucose peak levels were obtained in the pH range of 3.5 to 5.0.

EXAMPLE VII

30 Solutions containing substrate (MD03, 33% DS) were adjusted to pH 4.2 and heated to various temperatures. Amyloglucosidase (21 AGI/gDS) and a 9-fold amount of acid amylase were added. Aliquots were taken and analyzed as described in Example II. Controls (AG dosages without extra 35 AA addition) were also carried out. The results are given in Table VI below.

TABLE VI

Temperature	AAU/AGI	saccharification time in hr.	glucose yield (%)
55	0.074	65	94.6
	0.74	47	95.0
57.5	0.074	71	95.0
	0.74	47	95.5
60	0.074	71	94.8
	0.74	64	95.3
62.5	0.074	90	94.8
	0.74	64	95.4
65	0.074	117	93.0
	0.74	89	94.6

15

These results confirm that acid amylase is stable at temperatures up to at least 65°C, which makes it very suitable for use in conjunction with amyloglucosidase at relatively high saccharification temperatures. The lower glucose values at 65°C are likely caused by the lower thermostability of the AG enzyme relative to AA. The presence of acid amylase has a beneficial effect on the glucose production at higher temperature.

25

CLAIMS

1. A microbial acid amylase having substantially  $\alpha$ -1,4-glucosidic bond splitting activity.
- 5 2. A microbial acid amylase showing optimum saccharification at a pH from 3.5 to 5.0 at a temperature from 60 to 75°C.
3. The acid amylase of claim 1 or 2 which is derived from a fungus.
- 10 4. The acid amylase of claim 3 which is derived from Aspergillus niger.
5. An enzyme product comprising amyloglucosidase and acid amylase as defined in any one of claims 1 to 4 in a ratio of at least 0.16 AAU per AGI, as hereinbefore defined.
- 15 6. An enzyme product according to claim 5 containing 0.2 to 4.5 AAU per AGI.
7. An enzyme product according to claim 5 or 6 wherein the amyloglucosidase is derived from Aspergillus niger.
- 20 8. An enzyme product according to any one of claims 5 to 7, substantially free of transglucosidase.
9. An enzyme product according to any one of claims 5 to 8 also containing an effective amount of acid pullulanase.
- 25 10. An enzyme product according to claim 9 wherein the acid pullulanase is a pullulanase as described in European Patent Application Publ. No. 0 063 909.
11. A process for converting starch into dextrose in the form of a syrup which comprises saccharifying starch or a  
30 starch hydrolysate in the presence of an enzyme product as defined in any one of claims 5 to 10.
12. A process according to claim 11 wherein a starch hydrolysate containing at least 30% by weight of dry solids is saccharified.
- 35 13. A process according to claim 11 or 12 wherein the saccharification is conducted in the pH-range of from 3 to 5 and at a temperature in the range of from 40 to 70°C.
14. A process according to claim 13 wherein the

saccharification is conducted at pH 4 to 4.5 at a temperature of 50 to 65°C.

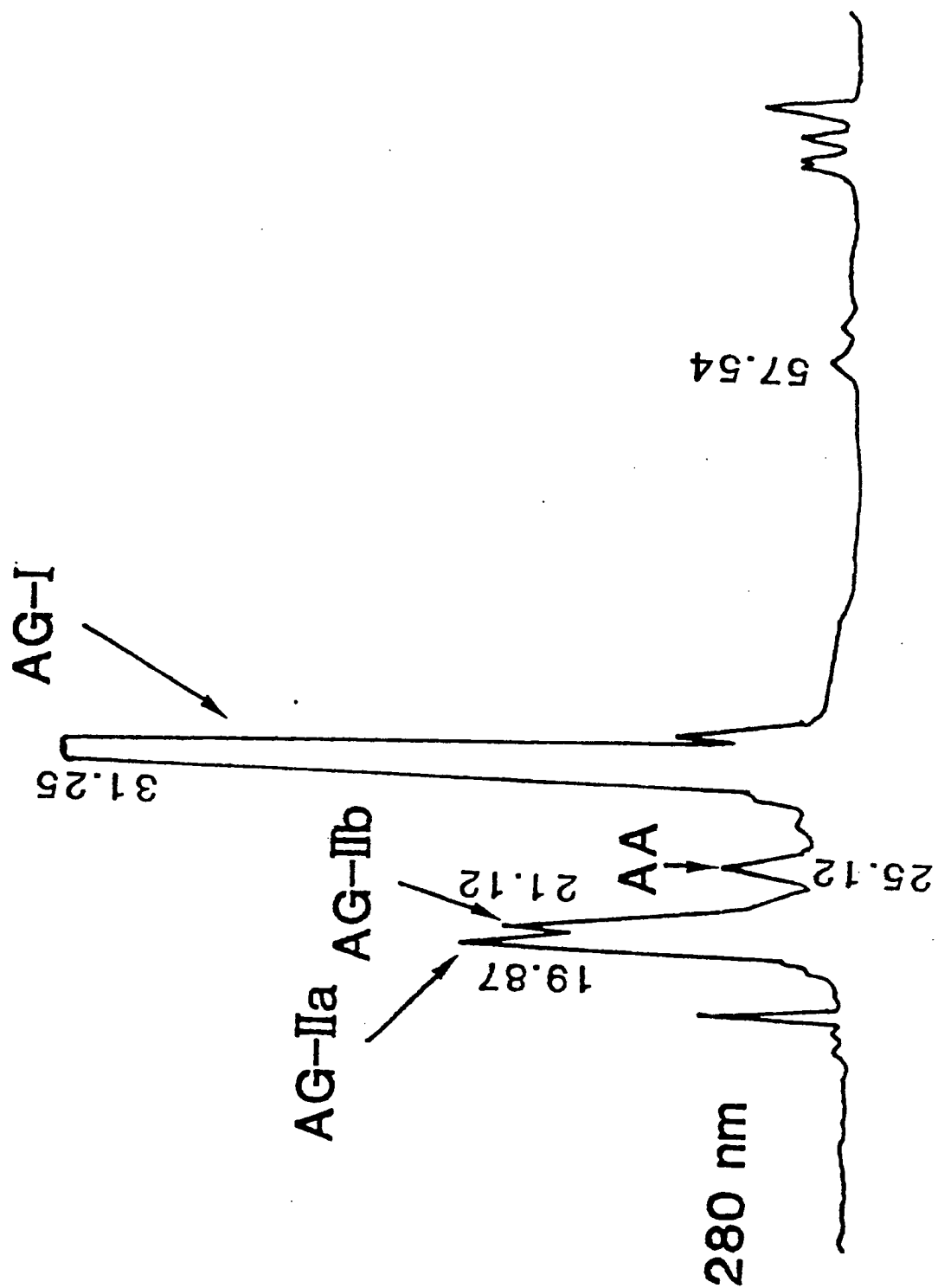
15. A process according to any of claims 11 to 14 wherein the amount of amyloglucosidase used is from 8 to 30 5 AGI per g of total dry solids.

16. A process according to claim 15 wherein the amount of amyloglucosidase used is from 14 to 22 AGI per g of total dry solids.

17. A process according to any one of claims 11 to 16 10 wherein the saccharification is conducted in the presence of acid pullulanase.



11

FIG. I



European Patent  
Office

# EUROPEAN SEARCH REPORT

0140410

Application number

EP 84 20 1301

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X	DIE STÄRKE, vol. 27, no. 11, November 1975, pages 377-383, Weinheim, DE; J.J. MARSHALL: "Starch-degrading enzymes, old and new" * Page 381, left-hand column, chapter 4 - page 382, left-hand column, paragraph 1; page 378, right-hand column, lines 17-19 *	1-4	C 12 N 9/30 C 12 N 9/34 C 12 P 19/20
X	--- STARCH/STÄRKE, vol. 30, no. 8, 1978, pages 272-275, Verlag Chemie GmbH, Weinheim, DE; N. RAMACHANDRAN et al.: "Studies on the thermophilic amylolytic enzymes of a strain of Aspergillus niger" * Page 273, left-hand column, last paragraph - page 274, right-hand column *	1-4	
A	--- CHEMICAL ABSTRACTS, vol. 73, no. 17, 26th October 1970, page 35, no. 84092z, Columbus, Ohio, USA; J.J. MARSHALL et al.: "Incomplete conversion of glycogen and starch by crystalline amyloglucosidase and its importance in the determination of amylaceous polymers" & FEBS (FED. EUR. BIOCHEM. SOC.) LETT. 1970, 9(2), 85-88 (Cat. D) * Abstract *	1,3,4	C 12 N C 12 P C 12 D
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
Place of search THE HAGUE		Date of completion of the search 13-12-1984	Examiner DEKEIREL M.J.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			



European Patent  
Office

# EUROPEAN SEARCH REPORT

0140410

Application number

EP 84 20 1301

Page 2

DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)	
A	<p>STARCH/STÄRKE, vol. 30, no. 10, 1978, pages 341-345, Verlag Chemie GmbH, Weinheim, DE; P. FINCH et al.: "Comparative studies on glucoamylases isolated from a strain of Aspergillus"</p> <p>* Page 344, left-hand column *</p>	1		
A	<p>--- CHEMICAL ABSTRACTS, vol. 96, no. 3, 18th January 1982, page 208, no. 17141b, Columbus, Ohio, USA; G.I. KVESITADZE et al.: "Selection of microscopic fungi producing acid-stable alpha-amylase and glucoamylase" &amp; MIKROBIOLOGIYA 1981, 50(5), 807-812</p> <p>* Abstract *</p>	1		
A	<p>--- GB-A-1 037 710 (CORN PRODUCTS) * Page 3, lines 60-71, 128-130; page 4, lines 1-48; page 4, line 80 - page 5, line 75 *</p>	1,3,4		TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
A	<p>--- GB-A-2 016 476 (HIRAM WALKER &amp; SONS) * Claims 1,8 *</p>	1,3,4		
A	<p>--- US-A-4 284 722 (M. TAMURI et al.) * Example 5; column 19, lines 22-29; column 20, lines 11-44 *</p> <p>--- -/-</p>	1		
The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 13-12-1984	Examiner DEKEIREL M.J.	
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>				



European Patent  
Office

# EUROPEAN SEARCH REPORT

0140410

Application number

EP 84 20 1301

DOCUMENTS CONSIDERED TO BE RELEVANT			Page 3
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D, A	EP-A-0 063 909 (NOVO INDUSTRI)		
	---		
A	GB-A-1 106 421 (K.F. MELLOR)		
	---		
A	US-A-4 017 363 (W.H. McMULLEN et al.)		
	---		
A	DIE STÄRKE, vol. 27, no. 4, 1975, pages 123-128, Weinheim, DE; S. UEDA et al.: "Multiple forms of glucoamylase of rhizopus species"		
	-----		
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 13-12-1984	Examiner DEKEIREL M.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document	